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EVALUATION OF A RECOMBINANT *ESCHERICHIA COLI* STRAIN THAT USES THE SARIN SIMULANT ISOPROPYLMETHYLPHOSPHONIC ACID (IMPA) AS A SOLE CARBON AND PHOSPHATE SOURCE

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14. ABSTRACT:

Chemical nerve agents consist of organophosphates that irreversibly inhibit the enzyme acetylcholinesterase within minutes, resulting in death by asphyxiation unless immediate medical support is provided. Currently, different methods for the destruction of large stockpiles of these agents are under evaluation. A method of destruction proposed by the Lawrence Berkeley National Laboratory (LBNL; Berkeley, CA) sought the development of a recombinant *Escherichia coli* strain that utilizes the nerve agent sarin as a sole carbon and phosphate source. To ascertain whether these recombinant strains degrade sarin, the Defense Advanced Research Projects Agency (Arlington, VA) requested that the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) evaluate the LBNL-developed recombinant strains for efficacy. In this study, only phosphate use by these recombinant strains was evaluated because carbon use by these strains is still undergoing optimization by LBNL. The *E. coli* strains produced by LBNL yielded little growth on the sarin simulant isopropylmethylphosphonic acid (IMPA). However, ECBC-transformed strains, using plasmids, had successful growth when transformed into a different *E. coli* background, which correlated with IMPA degradation. Ultimately, the transformed *E. coli* strains, optimized at ECBC, were able to grow using IMPA as the phosphate source.

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PREFACE

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EVALUATION OF A RECOMBINANT *ESCHERICHIA COLI* STRAIN THAT USES THE SARIN SIMULANT ISOPROPYLMETHYLPHOSPHONIC ACID (IMPA) AS A SOLE CARBON AND PHOSPHATE SOURCE

1. INTRODUCTION

Chemical nerve agents are organophosphates that can irreversibly inhibit acetylcholinesterase (AChE) in minutes, resulting in death without immediate medical support (*1*). Once AChE is irreversibly inhibited, the body must produce more to resume normal function, which can take weeks. Chemical nerve agents include G-agents (soman, sarin) and V-agents. It can be difficult to safely destroy a large volume of toxic chemicals because it is best to degrade the byproducts as well as the chemical agents themselves. Past destruction methods have included incineration and deep-ocean dumping (*2*). In addition, chemical destruction has seen a recent success with Syria's chemical weapons stockpile, neutralized safely by members of the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) (*3*). However, alternative destruction methods are always under study.

One method proposed by the staff at Lawrence Berkeley National Laboratory (LBNL; Berkeley, CA) was the biological destruction of chemical nerve agents. Multiple enzyme classes were discovered that can detoxify organophosphates by hydrolysis, such as phosphotriesterases (ptes; also termed organophosphorus hydrolases) and organophosphorus acid anhydrolases (*2*). Although these enzymes were recombinantly engineered for increased effectiveness against chemical nerve agents, the resulting products still remained in high concentrations and needed to be removed. Therefore, an ideal decontamination scheme would involve the total degradation of chemical nerve agents into common small molecules in the environment. Similar efforts have been published in the past, such as activated sludge using sarin as a sole carbon source (*4*), *Escherichia coli* using the pesticide paraoxon as a sole phosphate source (*5*), and *Pseudomonas putida* using paraoxon as a sole carbon and phosphorus source (*6*). In the *P. putida* effort, a recombinant bacterial strain was synthetically engineered by adding a phosphodiesterase (pdeA) from *Delftia acidovorans*, a bacterial species capable of using some organophosphates as sole phosphate sources (*7*), and a phosphotriesterase from *Flavobacterium* sp. strain ATCC 27551. LBNL staff proposed using similar methods with recombinant *E. coli* and attempting to design a strain that could use sarin (isopropyl methylphosphonofluoridate, GB) as a sole carbon and phosphorus source.

The *phn* operon is native to *E. coli* and is part of the *pho* regulon, which is necessary for phosphate uptake and metabolism (*8*). This operon includes 14 cistrons, *C–P*. Genes *C–E* code for a phosphate ABC transporter, *phnF* is a repressor, genes *G–M* code for a C–P lyase (also expressed in pBbB2k), *phnN* is a phosphokinase, *phnO* is an *N*-acetyl transferase, and *phnP* is a phosphodiesterase. This operon is used by *E. coli* to utilize methylphosphonate as a sole phosphate source. However, the operon is turned off ("phase-off") at a high rate in K12 strains (e.g., DH10B) due to a repeat sequence (*9*), whereas *E. coli* B strains (e.g., BL21) are "phase-on". Other phosphate sources require additional phosphate transporters within the *pho* regulon.

Experimental efforts to use sarin as a carbon or phosphorus source were performed as two separate projects at LBNL. The planned degradation pathways are shown in Figure 1.

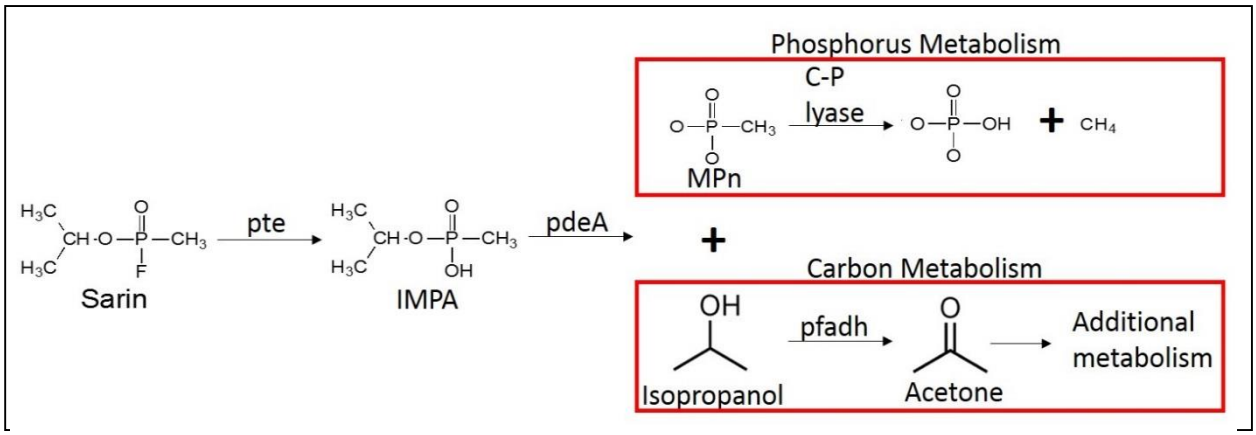

Figure 1. Proposed sarin degradation by recombinant *E. coli*. Abbreviations: pfadh (alcohol dehydrogenase), IMPA (isopropylmethylphosphonic acid), and MPn (methylphosphonic acid).

LBNL personnel developed two plasmids to encode all of the necessary enzymes for metabolizing sarin as a phosphorus source (Figure 2). The degradation of sarin to IMPA by pte requires recombinant expression, and the exact phosphotriesterase chosen for this effort by LBNL personnel is not described in the provided plasmid maps. However, the pte sequence in plasmid pBbA5a (Figure 2B) matches the enzymes from both *Brevundimonas diminuta* and *Flavobacterium* sp., but not with complete identity. In general, phosphotriesterases have broad substrate specificity (*2*), and recombinant enzymes were mutated to enhance activity toward chemical nerve agents, including sarin. The degradation of IMPA by pdeA also requires an exogenous enzyme source; therefore, LBNL personnel cloned pdeA from *D. acidovorans* into plasmid pBbA5a. All other phosphate-metabolism steps can be performed by endogenous enzymes in *E. coli*, including C–P lyase, which is an enzyme produced by the *phn* operon (*10*). LBNL personnel concluded that the native *E. coli phn* operon was not expressed at a level that was sufficient for phosphate utilization of sarin, and they designed plasmid pBbB2k (Figure 2A) to increase expression. The tetracycline promoter was not induced, and sufficient C–P lyase was produced through leaky expression (*11*).

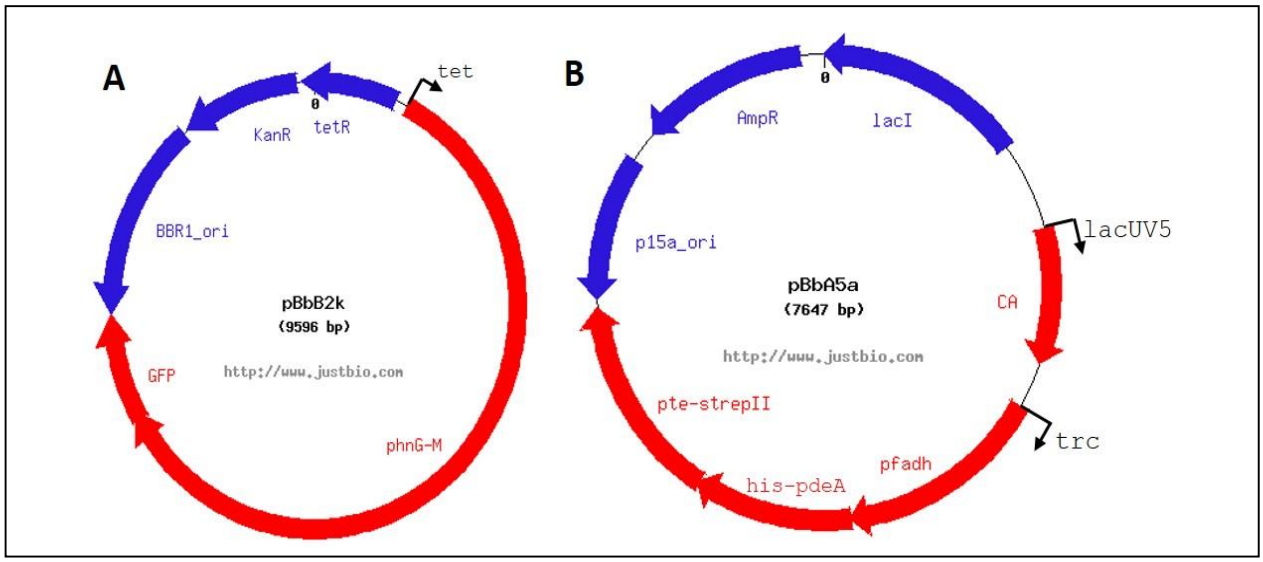

Figure 2. Plasmids developed by LBNL personnel for use of sarin as a sole phosphate source.

In Figure 2, plasmid A (pBbB2k) expresses a segment of the *phn* operon encoding for the *C–P* lyase from a tetracycline promoter. This operon includes green fluorescent protein (GFP) at the 3′ end of the putative transcript to confirm full transcription of the operon. Plasmid B (pBbA5a) expresses the enzymes necessary for both carbon and phosphorus utilization of sarin. CA (carbonic anhydrase), pfadh, pdeA (N-terminally his_6-tagged), and pte (C-terminally strep II-tagged). The plasmid pBbA5a comes in three versions: the full plasmid shown in Figure 2 (pBbA5a pdeA); a plasmid expressing pdeA H191A (pBbA5a pdeA H191A, which is catalytically dead according to LBNL personnel); and a plasmid with pte removed (pBbA5a pdeA Δpte).

The plasmid maps (Figure 2) were derived from plasmid files sent by LBNL personnel. These maps differ from the protocols sent from LBNL in two ways: (1) p15a origin of replication in plasmid B is listed as SC101, and (2) the trc promoter is not mentioned in plasmid B, and lacUV5 is listed as the promoter for an operon of four enzymes.

Many other enzymes required for sarin carbon metabolism, including pfadh, are not endogenous to *E. coli*. However, the plasmid pBbA5a pdeA (Figure 2B) includes pfadh and CA. Neither gene is necessary for phosphate metabolism of sarin, but both are expressed together, upstream of pdeA and pte, as a single operon. As with plasmid pBbB2k, the enzymes on plasmid pBbA5a pdeA are also not induced for phosphate use and rely on leaky expression. CA is listed on the plasmid map as being expressed by a promoter (lacUV5) separate from the other three genes; however, all four genes are listed as being under the control of the trc promoter in the LBNL protocols.

At the Defense Advanced Research Projects Agency's request, ECBC is evaluating the *E. coli* strains produced by LBNL to use sarin as a sole phosphate source. The original request was to evaluate sarin as a sole carbon source as well, but LBNL only sent ECBC the strain for phosphorus utilization because they wished to further optimize the carbon

utilization effort. Therefore, carbon metabolism efforts are not covered in this technical report. The items received from LBNL are listed in Table 1. All strains and plasmids used for this sutdy are listed in the appendix, Table A.1.

Table 1. Items Received from LBNL

Name	Item Type	Notes
Protocols	Microsoft Word file	Includes bacterial genotypes, plasmid details, growth protocols, and enzymatic activity protocols.
Plasmid maps	GB files	Annotated sequence files for all four plasmids.
Strain 20	Glycerol stock	*E. coli* DH10B transformed with plasmids pBbB2k and pBbA5a pdeA.
Strain 21	Glycerol stock	*E. coli* DH10B transformed with plasmids pBbB2k and pBbA5a pdeA H191A.
Strain 22	Glycerol stock	*E. coli* DH10B transformed with plasmids pBbB2k and pBbA5a pdeA Δpte.
Plasmids	Plasmid DNA	One tube each of the four plasmids.
IMPA	Chemical	LBNL personnel produced their own IMPA from a precursor because they were unable to obtain a commercial source.

2. METHODS

2.1 Bacterial Strains Used and Growth Conditions

The LBNL glycerol stocks (recombinant *E. coli* DH10B; genotype F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ– as per LBNL) were used, as well as *E. coli* BL21 T7 Express (genotype *fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10*--TetS*)2 [dcm] R(*zgb-210::Tn10--TetS) endA1 Δ(mcrC-mrr)114::IS10). Plasmid propagation was performed with *E. coli* DH5α.

Strains were grown in tryptic soy broth (TSB; Sigma-Aldrich Company; St. Louis, MO) or EZ Rich medium (Teknova, Inc.; Hollister, CA) at 37 °C with shaking, unless otherwise noted. EZ Rich medium was prepared with sterile Milli-Q water (18.2 mΩ; EMD Millipore; Billerica, MA). Additives and final media concentrations included kanamycin at 50 µg/mL and carbenicillin at 100 µg/mL (Sigma-Aldrich), isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 1 mM (Sigma-Aldrich), IMPA at 1.32 mM (Cerilliant Corporation; Round Rock, TX), and chemical agent standard analytical reference material (CASARM)-grade sarin at 1.32 mM (produced by ECBC).

2.2 Plasmid Purification and Sequencing

Plasmids were transformed into *E. coli* DH5α for propagation and purified with the QIAprep Spin Miniprep kit (Qiagen, Inc.; Hilden, Germany). Sequencing primers are listed in the appendix, Table A.2. Plasmids were Sanger-sequenced at the Synthesis and Sequencing Facility, Johns Hopkins University (Baltimore, MD).

2.3 Bacterial Strain Characterization

Single colonies were picked from TSB agar plates to start overnight cultures in EZ Rich medium. These cultures were centrifuged at 6000 × *g* for 5 min and washed three times with EZ Rich media that was lacking phosphate. The cultures were diluted 1:100 in Erlenmeyer flasks. At each time point, 1 mL of culture was removed for optical density at a wavelength of 600 nm (OD_{600}) measurement. The 1 mL samples were centrifuged at 6000 × *g* for 5 min, and pellets were saved at –80 °C for protein studies. Clarified media was diluted 1:100 in 1:1 high-performance liquid chromatography (HPLC)-grade water/methanol for mass spectrometry and stored at –80 °C.

2.3.1 Protein Analysis

Single colonies were picked from TSB agar plates to start overnight cultures in EZ Rich medium. The next day, cultures were diluted 1:10 in EZ Rich medium, grown to an OD_{600} of 0.4–0.6, and induced by adding 1 mM IPTG and placing the cultures at the appropriate temperature. After the appropriate length of time, the cultures were centrifuged at 6000 × *g* for 5 min and pellets were placed at –80 °C. Bacterial pellets were lysed with Bugbuster (EMD Millipore) in accordance with the manufacturer's directions. Total protein concentrations were determined using a Pierce bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Inc.; Waltham, MA). Protein samples were electrophoresed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose for western blotting. Blots were visualized with a LI-COR Odyssey system (LI-COR Biosciences; Lincoln, NE). Primary antibodies used were anti-mouse His_6 (Life Technologies; Grand Island, NY), anti-rabbit Strep II (Abnova Corporation; Taipei City, Taiwan), and anti-mouse GFP (Life Technologies). Secondary antibodies were donkey anti-rabbit 680RD and donkey anti-mouse 800CW (LI-COR Biosciences).

2.3.2 Mass Spectrometry Analysis

The analyte was methanol-extracted from the media by diluting the media 1:100 in HPLC-grade methanol. This was filtered using a 0.22 µm Costar filter (Corning Incorporated; Corning, NY) to remove any remaining bacteria. The filtered samples were dried in a speed-vac and resuspended in 100 µL of 88% acetonitrile (ACN) and 0.12 M ammonium fluoride, which was spiked with 9.1 ng/µL of internal standard (*12*). The internal standard was an isotopically labeled IMPA, isopropyl-d_7 (Sigma-Aldrich catalog no. ERI-017-1.2 mL). All samples were analyzed on an Agilent 1290 liquid chromatograph with an Agilent 6490 triple-quadrupole mass spectrometer as described by Hamelin and coauthors (*13*). Briefly, the analyte was resolved using liquid chromatography with a SeQuant ZIC-HILIC (hydrophilic interaction liquid

chromatography) HPLC column (EMD Millipore catalog no. 150461). The mobile phase was 100% ACN, and the aqueous phase was 100% H_2O with 1 mM ammonium fluoride. The flow was isocratic, 88:12% (ACN/H_2O), at 1.5 mL/min with an injection volume of 1 µL. The method ran for 2.5 min, and the retention time of the peak of interest was 0.998 min. The mass spectrometer was operated in multiple-reaction monitoring mode using electrospray ionization in negative ion mode. The instrument performed scans at 1.23 cycles/min or 814 ms per cycle. The first quad (Q1) and the third quad (Q3) were set to a resolution of 0.7 FWHM (full-width at half-maximum). In addition, 1500 Vdc was applied to the spray source nozzle, and 3000 Vdc was applied across the capillary. The nitrogen drying gas flow was 16 L/min controlled at 250 °C, and the sheath gas flow was 12 L/min and maintained at 350 °C. The precursors and transitions for IMPA and the internal standard are summarized in Table 2.

Table 2. Summary of Precursor Ions and Transitions

Analyte	Precursor (*m/z*)	Transition (*m/z*)	Collision Energy (V)
IMPA	137	94.9	10
IMPA-D_7	144	95.9	10

3. RESULTS

Initial culturing from the LBNL glycerol stocks yielded a less-than-optimal number of active colonies. Therefore, a single colony for each strain was cultured in TSB to produce a new glycerol stock, which was used in the resulting experiments. Plasmids were isolated from the LBNL glycerol stocks and then sequenced. Purified plasmids provided by LBNL personnel were also sequenced. All plasmids resulted in the correct sequences for all recombinant inserts of interest.

We used LBNL Strains 20 and 21 for initial growth tests with IMPA as a phosphorus source. Strain 20 is the experimental strain, which should be able to use sarin (or IMPA) as a sole phosphorus source. Strain 21 is a negative control that expresses catalytically dead pdeA, which should be unable to use IMPA as a phosphorus source. Strain 22 is a negative control that would be unable to degrade sarin due to the lack of pte but would be able to use IMPA. We grew the strains in EZ Rich media that lacked phosphate, in EZ Rich with K_2PO_4 as a phosphate source (standard EZ Rich media), or in EZ Rich with IMPA as a phosphate source. However, we were unable to replicate growth rates with the LBNL glycerol stocks as presented by LBNL (Figure 3).

Both strains were clearly viable (Figure 3A, purple growth curves), as shown by growth on normal EZ Rich medium. In addition, by Day 5, there was a definite growth difference between the two strains, as shown by the inset (OD_{600} = 0.169 vs 0.066 after 5 days). However, the LBNL-derived growth curve showed putative Strain 22 growth to OD_{600} of ~0.6 in 1 day. Potential explanations for the discrepancy include growth on ethyl methyl phosphonate versus IMPA or Strain 22 versus Strain 20 growth. We also compared commercially purchased IMPA with LBNL-produced IMPA as a potential variable. However, growth rates were similar

(data not shown). As a final consideration, LBNL personnel may have used a different *E. coli* background for this particular experiment.

Two different versions of the LBNL methods that were sent to ECBC listed the *E. coli* strain in use as DH1 (first version) or DH10B (second version). In addition, the genotype listed for the DH10B strain was for BL21(DE3). We concluded that we could not be fully certain of the *E. coli* strain actually in use. Therefore, we transformed the LBNL-produced plasmids of interest into a new background, BL21 T7 Express. This *E. coli* strain is *phn* operon phase-on, whereas DH1 and DH10B strains are phase-off.

Initial growth tests of the BL21 recombinant strains with IMPA were successful, and BL21 3+4 grew to an OD_{600} of greater than 1 in 5 days (Figure 4A). The BL21 1+4 strain grew to an OD_{600} of 0.5 in 5 days, and the negative control, strain BL21 2+4, showed negligible growth. All three strains grew equally in standard EZ Rich medium, and no strains grew in EZ Rich medium that lacked phosphate. These strains grew better than the original LBNL-provided strains, but still did not grow as well as that shown in Figure 3B. Therefore, we tested removal of the pBbB2k strain, which should not be necessary in a phase-on B strain. As shown in Figure 4B, these strains grew even better than the BL21 1+4 and BL21 3+4 strains and reached an OD_{600} of greater than 1 in 3–4 days for BL21-1 and BL21-3 strains. The negative control, BL21-2 strain, still showed negligible growth, as did all strains grown in media that lacked phosphate. Once again, all three strains grew equally well in the standard EZ Rich medium.

Because the BL21-1 strain grows well with IMPA as the phosphate source, we can assume that IMPA degradation is proceeding as shown in Figure 1. One way to measure this effect is by using mass spectrometry to determine the IMPA concentration in the media at each time point. As the OD_{600} increases, the IMPA concentration should decrease. IMPA concentrations were determined for the BL21-1, BL21-2, and BL21-3 strains during IMPA growth (Figure 5). The IMPA concentration decreased during BL21-1 and BL21-3 strain growth, but did not significantly change during BL21-2 strain growth. Approximately half of the IMPA remained after 5 days of growth with the BL21-1 and BL21-3 strains. Other IMPA metabolites were not measured, although that is possible using mass spectrometry as well.

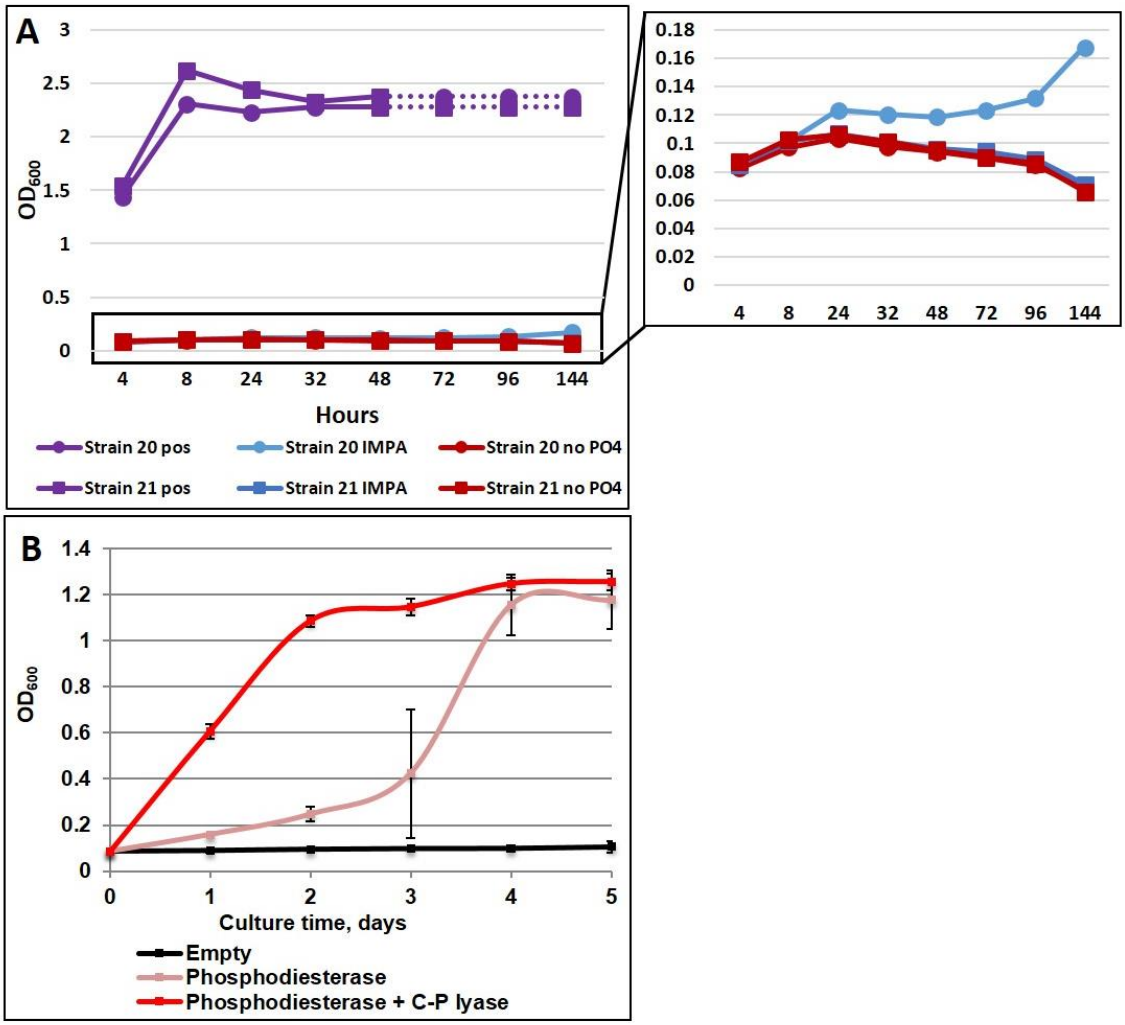

Figure 3. Growth rates of LBNL recombinant *E. coli* strains. (A) Strain 20 (experimental; circles) and strain 21 (negative control; squares) were grown on normal EZ Rich medium (purple), EZ Rich medium with IMPA (blue), or EZ Rich medium that lacked phosphate (red). The figure inset shows a close-up of IMPA and no phosphate growth curves. (B) The figure received by LBNL of their strains with an empty vector, pdeA expression, or pdeA, and C–P lyase expression that was grown in EZ Rich medium with ethyl methyl phosphonate as a phosphorus source.

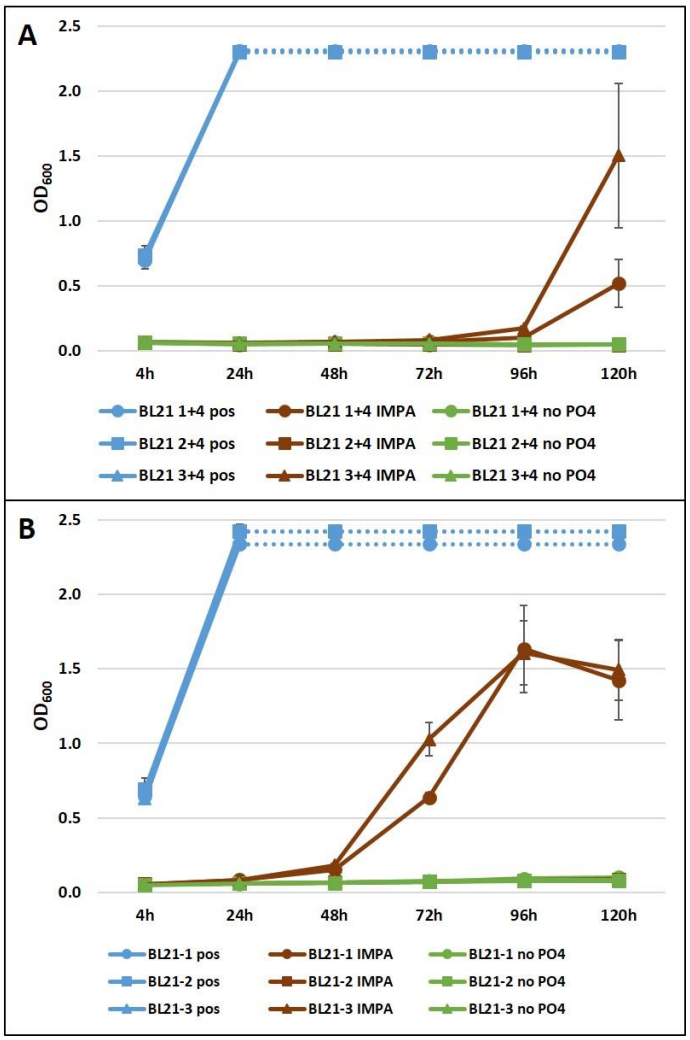

Figure 4. Growth rates of LBNL plasmids in strain BL21 T7 Express background. (A) *E. coli* BL21 T7 Express strains expressing both pBbB2k and pBbA5a plasmid versions. (B) *E. coli* strain BL21 T7 Express strains expressing only plasmid pBbA5a versions. Blue lines represent growth in complete EZ Rich medium, brown lines represent growth in EZ Rich medium with IMPA as a phosphorus source, and green lines represent growth in EZ Rich medium that lacks a phosphorus source. Circles represent strains expressing pdeA and pte; squares represent strains expressing pdeA H191A and pte; and triangles represent strains expressing pdeA and no pte. Values and statistics are shown in Table A.3. Statistics for pdeA H191A versus pdeA and pdeA Δpte were probability (p) < 0.05 and p < 0.005, respectively.

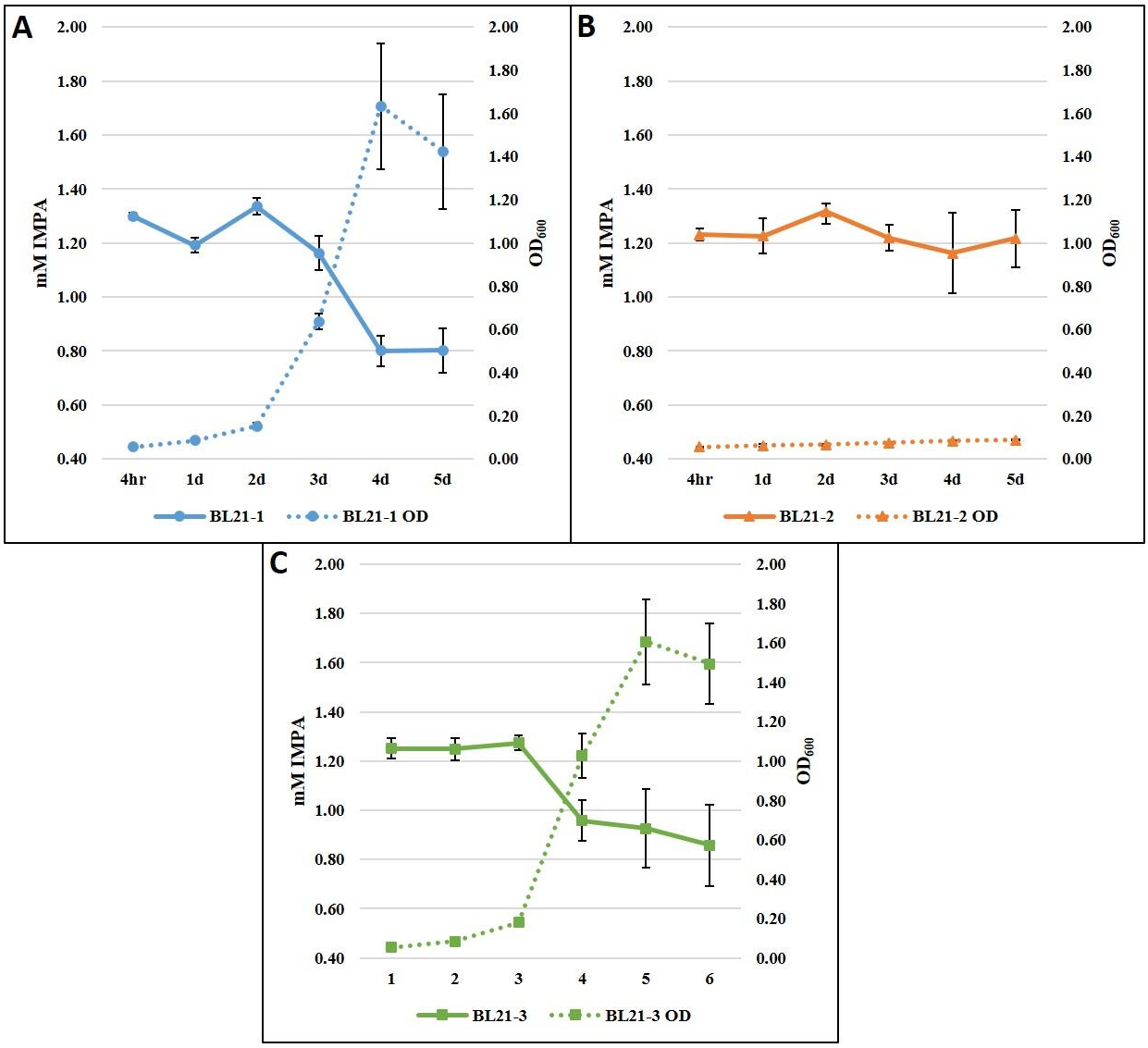

Figure 5. IMPA is degraded during microbial growth.
Media clarified by centrifugation was sampled at each time point and diluted 1:100 in 1:1 water/methanol. If IMPA is metabolized, the IMPA concentration decreases as microbial growth (OD_{600}) increases. Solid lines represent IMPA concentrations, and dotted lines represent OD_{600} values. (A) Strain BL21-1, (B) strain BL21-2, and (C) strain BL21-3. All time points are in triplicate with standard errors of the means.

The BL21-1, BL21-2, and BL21-3 strains still did not grow as readily with a sarin simulant as did the strain shown in Figure 3B. However, we determined that this level of growth was successful, and we used these strains to test growth on actual sarin. After three days of growth, the OD_{600} of the strains reached 1.67 ± 0.14 (BL21-1), 0.12 ± 0.02 (BL21-2), and 1.73 ± 0.11 (BL21-3). The BL21-1 and BL21-3 strains were significantly different from the BL21-2 strain ($p < 0.005$). Therefore, we saw equal growth in the BL21-1 and BL21-3 strains, which showed no difference whether the strains expressed the pte or not. We did not measure the

fluoride ion release in accordance with the statement of work (SOW) because of the high rate of sarin hydrolysis (Section 4), which was nearly complete before appreciable growth by any of the strains began. In addition, any hydrogen fluoride produced during this process would have been buffered by the 3-(*N*-morpholino)propanesulfonic acid (MOPS) in the media (1.32 mM sarin vs 40 mM MOPS). Therefore, we did not expect a pH change during growth. In conclusion, the negative control did not grow, which indicated that the BL21-1 and BL21-3 strains were successfully able to use sarin as a phosphorus source.

We also examined the protein expression from plasmids pBbA5a pdeA and pBbA5a pdeA Δpte. LBNL personnel were recently able to visualize the proteins of interest using a western blot but only in the insoluble fraction (*11*). We tested expression primarily in strains BL21-1 and BL21-3 because these strains were the simplest and lacked plasmid pBbB2k, which increased the chance of successful overexpression. We also tested induction at 24, 30, and 37 °C as difficult-to-express proteins may express better at lower temperatures. IPTG induction regularly results in overexpression that can be visualized in total protein stains. No overexpression was visualized at any molecular weight in the soluble fractions, as seen in the total protein stain (appendix, Figure A.1). Therefore, we western blotted for his_6 (pdeA tag) and strep II (pte tag). Western blots did not show either tag in the bacterial fractions, which implied that there was no induction of either pdeA or pte (data not shown). From these results, we concluded that a low level of pdeA must be expressed because strains BL21-1 and BL21-3 were able to use sarin as a phosphate source. However, the level was so low it could not be visualized by western blot. As indicated by the LBNL expression difficulties, the enzymes may be expressed in the insoluble fraction, or the synthetic operon may not transcribe or translate well. We did not perform enzyme activity analyses in accordance with the SOW because of the extremely low level of expression.

4. DISCUSSION

The primary question answered by this technical report was whether *E. coli* could be synthetically designed to use sarin as a sole phosphate source. The case is very strong to show that strains BL21-1 and BL21-3 can use sarin as a sole phosphorus source, as we were able to show statistically significant growth differences between strains BL21-1 and BL21-3 grown with sarin versus two different negative controls, strain BL21-2 (pdeA mutant) and all strains grown on media that lacked phosphate. The mutation-based negative control was chosen instead of an empty vector control, which showed that a lack of growth would not be due to toxic protein expression. The media that lacked phosphate was necessary as a negative control to confirm that bacterial growth was not due to residual phosphate from the overnight culture or from low phosphate levels remaining in the media reagents. We cannot fully conclude that strains BL21-1 and BL21-3 can use sarin as a sole phosphate source without radiolabeling to confirm incorporation of phosphorus derived from sarin; however, these controls make a strong case.

We did not see a significant growth difference between strains BL21-1 and BL21-3 when they were grown with sarin as the phosphorus source. The LBNL hypothesis was that strains expressing the pBbA5a pdeA plasmid protein would be able to grow on sarin, but strains expressing pBbA5a pdeA Δpte plasmid would not. This is because the pte, which

degrades sarin into IMPA, is not expressed in the pBbA5a pdeA Δpte plasmid. However, sarin is fairly labile when diluted in water and will hydrolyze by different mechanisms, depending on whether the solution is acidic or basic (*14*). We can calculate the hydrolysis rate of sarin in our proposed media solution based on the equation log $t_{1/2}$ = (5039/*T*) – 8.035 – pH, where *T* is in kelvin and the half-life, $t_{1/2}$, is in hours. In EZ Rich medium (pH 7.4) at 37 °C, the half-life of sarin is ~6.5 h. Therefore, 90% of the sarin is hydrolyzed into IMPA after 3.5 half-lives or after less than 24 h. Because these strains do not grow appreciably until at least 48 h after the initiation of the culture, the sarin hydrolysis rate is fast enough to have little impact on growth. If we were able to optimize the strains to use sarin as a phosphate source at the same rate as the use of K_2PO_4, pte expression may come into question.

Although we did not use the strains produced by LBNL for the entirety of this study, we did test their plasmids, which can be successfully used to allow *E. coli* to use sarin as a phosphate source. Because the LBNL growth curves showed a rate higher than all of the ECBC optimization attempts, we believe they may have used a different *E. coli* strain to produce those graphs as compared with the strains sent to ECBC. As mentioned in Section 1, *phn* operon phase-off strains are unable to use phosphonates as sources of phosphorus. This may explain why LBNL developed a plasmid that expressed portions of the *phn* operon when *E. coli* K12 strains were used as the background for this project. However, the *phn* operon includes not only the enzymes necessary for the degradation of phosphonates but also the necessary transporter in genes *C–E*. The transporter was not cloned into plasmid pBbB2k, so it is surprising that this plasmid would enhance IMPA utilization in an *E. coli* phase-off strain as shown in Figure 3B. However, LBNL personnel used ethyl methyl phosphonate as a phosphate source in the results shown in Figure 3B, whereas we used IMPA as the sarin simulant. This phosphonate source should still require *phn* operon expression, but this may still be a possible explanation for the growth-rate discrepancy.

We were surprised that the methods did not include induction of the relevant genes. Both the tet promoter and lac promoters (trc and lacUV5) are strong promoters with good repression. Therefore, we were surprised by the LBNL reliance on "leaky" expression. Although all promoters leak to some extent, this is more common with high-copy vectors, such as those with ColE1 as the origin of replication (*15*). The LBNL vectors use low-copy origins of replication; therefore, any "leakiness" would be reduced. In our case, phnG-M overexpression was not necessary, so any potential leaky expression was not an issue for this analysis. However, we did attempt to overexpress pdeA and pte via the trc promoter without success. There is experimental evidence that pte expression downstream of pdeA can reduce the activity of both (*6*), but we could not even detect pdeA overexpression in strain BL21-3, which lacked pte. Perhaps expression was affected by CA and pfadh, which were upstream of pdeA. However, uninduced growth of strains BL21-1 and BL21-3 shows that sufficient leaky expression was produced for bacterial survival, although the explanation for this is unclear.

It is unclear why strains BL21-1 and BL21-3 had a long lag phase before beginning exponential growth (Figure 4). However, similar results were observed by de la Pena et al. (*6*) when they used a similar system in *P. putida*. This result may have been caused by the slow rate of intracellular transport by organophosphates in bacteria (*16*) as well as the low expression of pdeA. IMPA degradation only reached ~50% after 5 days of growth with

strains BL21-1 and BL21-3 and appeared to plateau on Days 4–5. This may have been due to limiting carbon on those final days of growth. Therefore, nutrient conditions would need to be optimized to reach the goal of fully metabolizing IMPA and sarin.

5. FUTURE DIRECTIONS

The bioengineering in these strains shows some promise. Therefore, this section contains a few suggestions for future consideration should this work continue. We recommend the use of Dr. Jay Keasling's cited *P. putida* strain, which uses paraoxon as a sole carbon and phosphorus source (*6*). This control would be very relevant for comparison with any expression or enzymatic activity difficulties in *E. coli*. For phosphate utilization, there are a few procedural changes we suggest. We would attempt to overexpress only pdeA and pte in *E. coli* and remove CA and pfadh. This would focus work on phosphate utilization and potentially increase expression and the growth rate. We would work toward measurable overexpression, which should not require a western blot. At that point, we would expect better growth on sarin. However, the rate-limiting step for bacterial degradation of nerve agents is intracellular import (*16*). For this reason, multiple studies express organophosphate-degrading enzymes in the periplasmic space or cell surface (*17*). This system should be tested for these efforts, primarily for pte and pdeA expression and potentially for other enzymes, to allow for successful import of phosphate by the bacterium. In addition, *P. putida* organophosphate import should be analyzed to understand some of the differences between this bacteria and *E. coli*.

The carbon utilization by bacteria is more difficult to determine, and at the time of this report, LBNL was still optimizing this effort. All living organisms require the elements carbon, nitrogen, oxygen, and hydrogen in large quantities and phosphorus in smaller quantities. If sarin import limits phosphate use by bacteria, the slow rate of phosphate metabolism would affect carbon utilization at a greater level. These suggestions may allow for sufficient import of phosphate by the bacteria so that further optimization is not necessary to study carbon utilization. However, enzymatic expression is also under study, and we do not have sufficient information from LBNL to make further suggestions.

In addition, it may be useful to focus on the degradation of a different nerve agent as the hydrolysis rate of sarin in water is relatively rapid. Therefore, only a low amount of pte would be necessary for use in decontaminating a chemical stockpile scenario. All G- and V-agents hydrolyze in water relatively rapidly, so the metabolic focus may be of better use at the later metabolism steps.

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ACRONYMS AND ABBREVIATIONS

AChE	acetylcholinesterase
ACN	acetonitrile
BCA	bicinchoninic acid
CA	carbonic anhydrase
CASARM	chemical agent standard analytical reference material
ECBC	U.S. Army Edgewood Chemical Biological Center
FWHM	full-width at half-maximum
GB	isopropyl methylphosphonofluoridate, sarin
GFP	green fluorescent protein
HPLC	high-performance liquid chromatography
IMPA	isopropylmethylphosphonic acid
IPTG	isopropyl β-D-1-thiogalactopyranoside
LBNL	Lawrence Berkeley National Laboratory
MOPS	3-(*N*-morpholino)propanesulfonic acid
MPn	methylphosphonic acid
OD_{600}	optical density at a wavelength of 600 nm
p	probability
pdeA	phosphodiesterase (enzyme)
pfadh	alcohol dehydrogenase
pte	phosphotriesterase (enzyme)
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOW	statement of work
TSB	tryptic soy broth

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APPENDIX

STRAINS AND PLASMIDS

Table A.1. Strains and Plasmids Used In This Report

Strain or Plasmid Name	Description	Reference
pBbB2k	Plasmid containing *PHN G–M*; Kan^R, BBR1 ori	LBNL
pBbA5a pdeA	Plasmid containing CA, pfadh, pdeA, pte; Amp^R, p15a ori	LBNL
pBbA5a pdeA H191A	Plasmid containing CA, pfadh, pdeA H191A (catalytically dead), pte; Amp^R, p15a ori; negative control for IMPA	LBNL
pBbA5a pdeA Δpte	Plasmid containing CA, pfadh, pdeA; Amp^R, p15a ori; no pte, negative control for sarin	LBNL
Strain 20	*E. coli* DH10B; pBbB2k and pBbA5a pdeA	LBNL
Strain 21	*E. coli* DH10B; pBbB2k and pBbA5a pdeA H191A	LBNL
Strain 22	*E. coli* DH10B; pBbB2k and pBbA5a pdeA Δpte	LBNL
BL21 1 + 4	*E. coli* BL21 T7 Express; pBbB2k and pBbA5a pdeA	This work
BL21 2 + 4	*E. coli* BL21 T7 Express; pBbB2k and pBbA5a pdeA H191A	This work
BL21 3 + 4	*E. coli* BL21 T7 Express; pBbB2k and pBbA5a pdeA Δpte	This work
BL21-1	*E. coli* BL21 T7 Express; pBbA5a pdeA	This work
BL21-2	*E. coli* BL21 T7 Express; pBbA5a pdeA H191A	This work
BL21-3	*E. coli* BL21 T7 Express; pBbA5a pdeA Δpte	This work
BL21-4	*E. coli* BL21 T7 Express; pBbB2k	This work
DH5α-1	*E. coli* DH5α; pBbA5a pdeA	This work
DH5α-2	*E. coli* DH5α; pBbA5a pdeA H191A	This work
DH5α-3	*E. coli* DH5α; pBbA5a pdeA Δpte	This work
DH5α-4	*E. coli* DH5α; pBbB2k	This work

LBNL, Lawrence Berkeley National Laboratory; Kan^R, kanamycin resistance; ori, origin of replication, CA, carbonic anhydrase; pfadh, alcohol dehydrogenase; pdeA, phosphodiesterase; pte, phosphotriesterase; Amp^R, ampicillin resistance.

Table A.2. Primers Used In This Report

Primer Name	Sequence (5′→3′)	For plasmid	Gene Sequenced
CA-F2	CCCAGGCTTTACACTTTATGC	pBbA5a	CA
CA-R	CGTTATGATGTCGGCGCAAA	pBbA5a	CA
pfadh-F1	TTTGCGCCGACATCATAACG	pBbA5a	pfadh
pfadh-F2	TGAGGGTGATGTGGTCGAAC	pBbA5a	pfadh
pdeA-int	TTCATCTGATTCTCGGCAACC	pBbA5a	pte
pte-int	CGGTAGCCGCAAAGCTCTAG	pBbA5a	pte
pte-R	TAGCGAGTCAGTGAGCGAGG	pBbA5a	pte
phnG	GATTCCGACCTCATTAAGCAGC	pBbB2k	phnG
phnH	GTTCGCGGAGACAACGCATG	pBbB2k	phnH
phnI-1	GTGCTGATGTACGTTGCCGTG	pBbB2k	phnI
phnI-2	GCGGCGACGAAGGCTATTTG	pBbB2k	phnI
phnJ-int	CCGGTTATCAGGTGCCGTTTGG	pBbB2k	phnJ
phnK-1	CCGTTCAGCAGTGGGATGAG	pBbB2k	phnK
phnK-2	CCGAAGCTGGTGTTTATGGATG	pBbB2k	phnK
phnL-int	GATCAAACACGGTGACGAGTGGG	pBbB2k	phnL
phnM-int	GGTGCAGAACGGCGAAATCC	pBbB2k	phnM
phnM-2	CACGCCCACGTTGCTGAATC	pBbB2k	phnM
phnM-3	GGCGCATCGCAAGGACAATC	pBbB2k	phnM
GFP-int	GAAGATGGCTCCGTTCAACTAG	pBbB2k	GFP

Table A.3. OD_{600} for Strains Grown In IMPA (from Figure 4)

Strain	4 h	24 h	48 h	72 h	96 h	120 h
BL21 1+4	0.063 ± 0.002	0.058 ± 0.005	0.066 ± 0.005*	0.07 ± 0.007*	0.099 ± 0.021*	0.52 ± 0.18*
BL21 2+4	0.067 ± 0.004	0.054 ± 0.011	0.054 ± 0.005	0.049 ± 0.006	0.049 ± 0.006	0.049 ± 0.006
BL21 3+4	0.063 ± 0.002	0.06 ± 0.003	0.069 ± 0.005*	0.083 ± 0.009**	0.171 ± 0.02**	1.5 ± 0.556*
BL21-1	0.056 ± 0.002	0.085 ± 0.006*	0.15 ± 0.015**	0.636 ± 0.036**	1.63 ± 0.292**	1.42 ± 0.267**
BL21-2	0.055 ± 0.003	0.062 ± 0.005	0.07 ± 0.004	0.07 ± 0.005	0.083 ± 0.004	0.088 ± 0.002
BL21-3	0.054 ± 0.004	0.085 ± 0.005*	0.18 ± 0.014**	1.03 ± 0.113**	1.6 ± 0.215**	1.5 ± 0.204**

Statistics are BL21 2+4 vs BL21 1+4 or BL21 3+4; or BL21-2 vs BL21-1 or BL21-3.
*$p < 0.05$
**$p < 0.005$

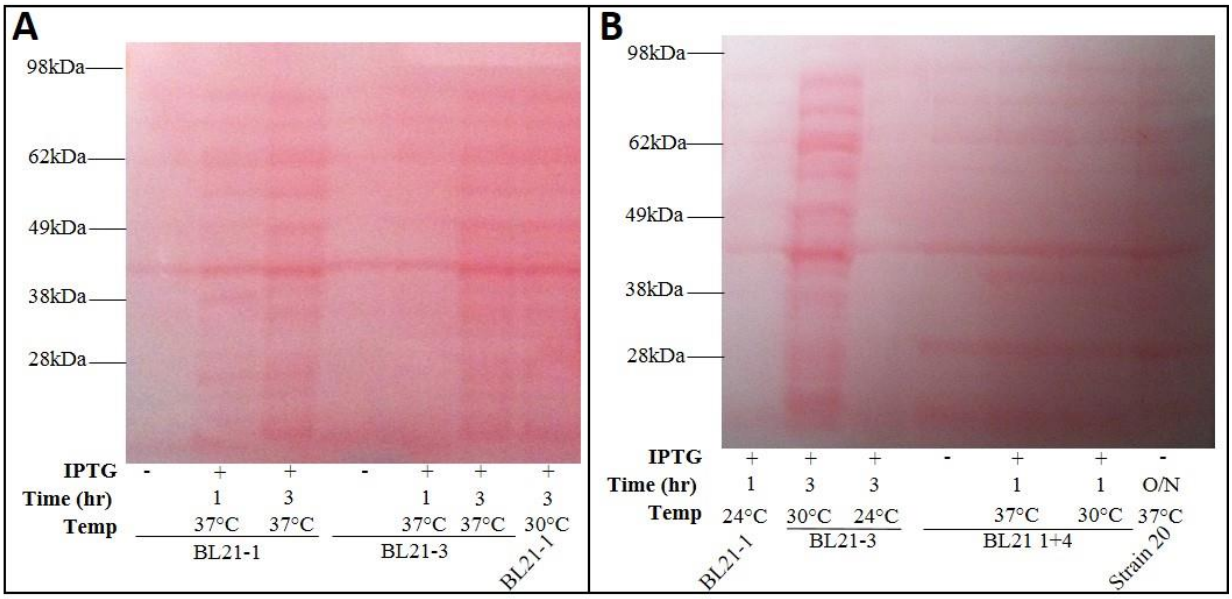

Figure A.1. Ponceau S protein expression study. *Escherichia coli* strains were grown in EZ Rich medium and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG), which should induce all four enzymes in plasmid pBbA5a. Induction was tested at multiple temperatures and for either 1 or 3 h. Protein lysates were electrophoresed and transferred, and total protein was stained with Ponceau S before western blotting. Expected molecular weights of proteins of interest were: CA (55 kDa), pfadh (88 kDa), pdeA (68 kDa), and pte (86 kDa).

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